PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

AAV DNA COMPRISING HELPER VIRUS SEQUENCES

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This is a national phase filing of the Application No. PCT/DE97/02500, which was filed with the Patent Corporation Treaty on October 24, 1997, and is entitled to priority of the German Patent Application 196 44 500.0, filed October 25, 1996.

I. FIELD OF THE INVENTION

The present invention relates to AA V DNA having helper virus sequences, a system containing such a DNA and its use.

II. BACKGROUND OF THE INVENTION

AAVs (adeno-associated viruses) are single stranded DNA viruses belonging to the Parvovirus family. For their replication, i.e. for forming viral particles, AAVs require helper viruses, particularly adenoviruses or herpesviruses. In the absence of helper viruses, AAVs may incorporate into the host cell genome, particularly at a specific site of chromosome 19.

The genome of AAVs is linear and has a length of about 4680 nucleotides. It comprises two reading frames which code for a structural gene and a non-structural gene. The structural gene is referred to as cap gene. It is controlled by the P40 promoter and codes for three capsid proteins. The non-structural gene is referred to as rap gene and codes for the Rep proteins Rep 78, Rep 68, Rep 52 and Rep 40. The two former proteins are expressed under the control of the P5 promoter, while the expression of Rep 52 and Rep 40 is controlled by the P19 promoter. The functions of the Rep proteins are represented *inter alia* by the control of replication and transcription of the AAV genome.

It has now turned out that preparations of recombinant (r)AAV viral particles are frequently contaminated with helper viruses, *e.g.*, adenoviruses or herpesviruses. This contamination considerably limits the use of rAAV viral particles for gen therapy. Efforts made to remove the helper viruses by CsC1 density gradient centrifugation or filtration

methods have produced little success so far, in particular these methods comprise steps which manifest themselves negatively as regards costs and yield.

Therefore, it is the object of the present invention to provide a product by which rAAV viral particles can be provided without contamination with helper viruses.

III. SUMMARY OF THE INVENTION

The present invention relates to an AAV DNA having helper virus sequences which are necessary for developing AAV viral particles, a system containing such a DNA and the use of both.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows the cloning strategy for obtaining the pTG9585 AAV DNA according to the invention.

Fig. 2 shows the cloning strategy for obtaining the pDG AAV DNA according to the invention.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a product by which rAAV viral particles can be provided without contamination with helper viruses. According to the invention this is achieved by the subject matters defined in the claims.

Thus, the subject matter of the present invention relates to an AAV DNA having helper virus sequences which are necessary for developing AAV viral particles.

The present invention is based on the applicant's finding that an AAV DNA according to the invention serves for inducing an rAAV vector commonly present in cells and containing a foreign DNA to develop rAAV viral particles without having to add helper viruses for this purpose.

The expression "AAV DNA" comprises any AAV DNA which may contain helper virus sequences necessary to develop AAV viral particles.

The expression "helper virus sequences" concerns any sequences of a helper virus necessary to dev lop AAV viral particles. Such sequences originat particularly from

herpesviruses and/or adenoviruses, more particularly from adenovirus 5. The sequences may comprise the entire virus genome or fragments thereof.

The expression "rAAV vector" comprises any AAV viral particle and its DNA, which may contain a foreign DNA, except for that of a helper virus, which is necessary to develop AAV viral particles.

With the above exception, the expression "foreign DNA" relates to any DNA which can be incorporated in an AAV vector. The foreign DNA can be non-coding or coding. In the former case, it may be a control element of DNA replication and/or transcription. In the latter case, it is favorable for the foreign DNA to be expressible, it being particularly advantageous for the expression to be controlled by an inducible promoter such as a tissue-specific promoter. In addition, the foreign DNA may code for a diagnostic and/or therapeutic protein. Examples of a therapeutic protein are tumor necrosis factor, plasma proteins and receptors. Moreover, the foreign DNA may be inserted at any site of the AAV vector.

An AAV DNA according to the invention can be prepared by common methods. By way of supplement, reference is made to Sambrook, J. et al., Molecular Cloning, A Laboratory Handbook (Vols. 1-3), Cold spring Habour, New York, (1989). Furthermore, reference is made, in Example 1, to the preparation of the pTG9585 AAV DNA according to the invention. This AAV DNA comprises the complete adenovirus 5 sequence with the exception of the E1 region, as helper virus sequences. PTG9585 is preferred. It was deposited with the DSM [German-type collection of micro-organisms and cell cultures], Braunschweig, as plasmid pTG9585 under number DSM 11248 on October 18, 1996. Also, an AAV DNA according to the invention is preferred which differs from pTG 9585 in that it has a deletion in the structural gene L1 of the adenovirus 5 sequence, particularly in the region of nucleotides 16614-18669. This AAV DNA is referred to as pTG9585 Δ 16614-18669. Besides, an AAV DNA according to the invention is preferred which differs from pTG 9585 in that it comprises two deletions from a total of 18323 base pairs, one deletion relating to great portions of the adenovirus capsid genes and the other deletion relating to the E3 region of adenovirus. This AAV DNA is referred to as pDG and was deposited with the DSM as plasmid pDG under number DSM 11817 on October 15, 1997.

A further subject matter of the present invention relates to a system comprising the above elements, i.e. an AAV DNA, an rAAV vector and optionally a cell. The AAV DNA and/or the cell represent a complementation as regards the AAV sequences of the rAAV vector. The expression "cell" concerns any cell, particularly mammalian cell, which permit the absorption and multiplication of AAV.

By means of the present invention it is possible to provide rAAV viral particle preparations without having to use helper viruses. Therefore, the rAAV viral particle preparations are also free from helper viruses. This is shown particularly when the pDG AAV DNA according to the invention is used. Moreover, the rAAV viral particle preparations distinguish themselves in that they contain no AAV wild-type. They also represent a subject matter of the present invention.

rAAV viral particle preparations according to the invention are perfectly suited for the transduction of cells. It may be favorable for the preparations to be treated with a Dnase prior to their use, so that free AAV DNA is degraded. The cells in consideration are any cells which are present in a body or isolated from a body. Hence it is possible by the present invention to take measures for an *ex vivo* and *in vivo* gene therapy.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Preparation f the AAV DNA pTG9585 according to the invention

The cloning strategy for obtaining pTG9585 is shown in fig. 1. An MMTV LTR fragment from PUC8MMTV (Fasel *et al.*, 1982, *EMBO J.* 1:3-7) is inserted in the multiple cloning site of plasmid pBSSKII(+) (company of Stratagene). Then, 4235 pb of AAV2 sequence from pAV2 (1983, *Gene* 2:65-73) are inserted in this plasmid by means of a synthetic oligonucleotide adapter, which contain thc complete rep gene and cap gene as well as the AAV2 promoters p19 and p40. Thus, thc AAV2 promoter p5, which controls the expression of the Rep78 proteins and Rep68 proteins, respectively, is replaced in the resulting plasmid pBMA2 by the MMTV promoter. The complete expression cassette consisting of the MMTV-LTR and the AAV2 rep gene and cap gene is then inserted in the vector pAdRSVßgal in the place of the RSV-ßgal fragment (J. Clin. Invest. 90, 625-6300. The MMTV-AAV2 fragment is flanked in the thus resulting plasmid pAMA2 on both sides by adenoviral sequences (5': 0-1.0 map units; 3':9.4-18 map units).

By means of homologous recombination (Chartier *et al.*, 1996, *J. Virol.* 70:4805-4810) the MMTV-AAV2 fragment from pAMA2 is inserted in the plasmid pTG3602 (Chartier *et al.*, *supra*). Thus, the resulting plasmid pTG9585 contains the complete adenovirus 5 sequence, with the exception of the E1 region which is substituted by the MMTV-AAV2 fragment. PTG9585 represents an AAV DNA according to the invention.

B. Example 2: Preparation of the pTG9585 Δ 16614-18669 AAV DNA according to the invention

A deletion of nucleotides 10983-18670 (the values related to the adenovirus 5 sequence0 is inserted in the AAV DNA pTG9585 prepared in Example 1 by restriction digestion using RsrII. Thereafter, a subfragment including nucleotides 10963-16613 is inserted in the deleted DNA molecule again. Thus, the deletion comprises a range of 2056 bp (nucleotides 16614-18669) from structural gene L1 of adenovirus 5. The pTG9585 Δ 16614-18669 AAV DNA according to the invention is obtained.

C. **Example 3: Preparation of the pDG AAV DNA according to the invention**

A deletion of nucleotides 5528 - 23677 is inserted in the AAV DNA pTG9585 prepared in Example 1 by restriction digestion using ClaI and SgfI. This deletion (18149 base pairs) comprises great portions of the adenovirus 5 capsid gene and the VA region important for the formation of rAAV viral particles. This region (1704 base pairs) is added to the remaining ClaI/SgfI fragment of pTG 9585 again. For this purpose, the VA region of adenovirus 5 is amplified by means of PCR and provided with 5' ends and 3' ends, respectively, which are compatible with ClaI and SgfI, respectively, so that a VA fragment is obtained which can be ligated with the above ClaI/SgfI fragment. An AAV DNA pTG 9585 is obtained which includes a deletion of 16445 base pairs. This AAV DNA is referred to as pTG 9585 Δ 16645.

A further deletion is inserted in pTG 9585 Δ 16445. It relates to the adenovirus 5-E3 region and presents itself as an XbaI fragment comprising 1878 base pairs (30827-32705). For this purpose, pTG 9585 is subjected to Bg1I digestion and a Bg1I fragment (27097 - 37445) which comprises the adenovirus 5-E3 region, is isolated and cloned into pBSSKII (Stratagene). The resulting DNA molecule is subjected to XbaI digestion, so that the above XbaI fragment can be separated. The rest of the DNA molecule is religated and, after Bg1I digestion, the resulting Bg1I fragment which is lacking the adenovirus 5-E3 region is inserted in pTG 9585 Δ 16445 by homologous recombination. The pDG AAV DNA according to the invention is obtained.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.